

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE
in its capacity as elected Office

Date of mailing (day/month/year)
21 June 2001 (21.06.01)

International application No.
PCT/GB00/03773

Applicant's or agent's file reference
H MJ03257WO

International filing date (day/month/year)
02 October 2000 (02.10.00)

Priority date (day/month/year)
01 October 1999 (01.10.99)

Applicant

GREGORIADIS, Gregory et al

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:
21 March 2001 (21.03.01)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was

☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Pascal Piriou

Telephone No.: (41-22) 338.83.38

PCT

From the INTERNATIONAL BUREAU

**NOTIFICATION OF THE RECORDING
OF A CHANGE**

(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)

To:

GILL JENNINGS & EVERY
Broadgate House
7 Eldon Street
London EC2M 7LH
ROYAUME-UNI

Date of mailing (day/month/year) 02 April 2002 (02.04.02)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference HMJ03257WO	
International application No. PCT/GB00/03773	International filing date (day/month/year) 02 October 2000 (02.10.00)

1. The following indications appeared on record concerning: <input checked="" type="checkbox"/> the applicant <input checked="" type="checkbox"/> the inventor <input type="checkbox"/> the agent <input type="checkbox"/> the common representative		
Name and Address GREGORIADIS, Gregory Lipoxen Technologies Limited 2d Wimpole Street London W1N 7AA United Kingdom	State of Nationality CA	State of Residence GB
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	
2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning: <input type="checkbox"/> the person <input type="checkbox"/> the name <input checked="" type="checkbox"/> the address <input type="checkbox"/> the nationality <input type="checkbox"/> the residence		
Name and Address GREGORIADIS, Gregory Suite 23 Bloomsbury House 4 Bloomsbury Square London WC1A 2RP United Kingdom	State of Nationality CA	State of Residence GB
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	
3. Further observations, if necessary: 		
4. A copy of this notification has been sent to: <input checked="" type="checkbox"/> the receiving Office <input type="checkbox"/> the designated Offices concerned <input type="checkbox"/> the International Searching Authority <input checked="" type="checkbox"/> the elected Offices concerned <input type="checkbox"/> the International Preliminary Examining Authority <input type="checkbox"/> other:		

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer Sangeeta JAIYA Telephone No.: (41-22) 338.83.38
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PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

GILL JENNINGS & EVERY
Broadgate House
7 Eldon Street
London EC2M 7LH
ROYAUME-UNI

Date of mailing (day/month/year) 05 October 2001 (05.10.01)	
Applicant's or agent's file reference HMJ03257WO	IMPORTANT NOTIFICATION
International application No. PCT/GB00/03773	International filing date (day/month/year) 02 October 2000 (02.10.00)

1. The following indications appeared on record concerning: <input checked="" type="checkbox"/> the applicant <input checked="" type="checkbox"/> the inventor <input type="checkbox"/> the agent <input type="checkbox"/> the common representative		
Name and Address GREGORIADIS, Gregory Lipoxen Limited 2d Wimpole Street London W1N 7AA United Kingdom	State of Nationality CA	State of Residence GB
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	
2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning: <input type="checkbox"/> the person <input type="checkbox"/> the name <input checked="" type="checkbox"/> the address <input type="checkbox"/> the nationality <input type="checkbox"/> the residence		
Name and Address GREGORIADIS, Gregory Lipoxen Technologies Limited 2d Wimpole Street London W1N 7AA United Kingdom	State of Nationality CA	State of Residence GB
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	
3. Further observations, if necessary:		
4. A copy of this notification has been sent to: <div style="display: flex; justify-content: space-between;"> <div> <input checked="" type="checkbox"/> the receiving Office <input type="checkbox"/> the International Searching Authority <input checked="" type="checkbox"/> the International Preliminary Examining Authority </div> <div> <input type="checkbox"/> the designated Offices concerned <input checked="" type="checkbox"/> the elected Offices concerned <input type="checkbox"/> other: </div> </div>		

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer <div style="text-align: center;">R. Raissi</div>
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference HMJ03257W0	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/GB 00/ 03773	International filing date (day/month/year) 02/10/2000	(Earliest) Priority Date (day/month/year) 01/10/1999
Applicant LIPOXEN LIMITED et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☒ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

☐ the text is approved as submitted by the applicant.

☒ the text has been established by this Authority to read as follows:

LIPOSOME-ENTRAPPED DNA ORAL VACCINES

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 18 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the vaccine.
2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Formula III as referred to in claim 1 appears to be erroneous in that groups denoted "R13", "R14", "R17" and "R18", the atom or bond denoted "Y1" as well as the number denoted "p" therein are not referred to in the text of the claim. Therefore, it has not been possible to carry out a meaningful search into the state of the art on the basis of the claims as filed. A limited search has been carried out with respect to the whole claimed subject-matter (claims 1-20) based on the assumption that (i) said formula was to be replaced by formula II as referred to on description page 2 and (ii) said formula II corresponds to the formula II as referred to in claim 13.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

II. National Application No

PCT 00/03773

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K9/127 A61K48/00 C12N15/88

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, CHEM ABS Data, BIOSIS, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GREGORIADIS G ET AL: "Vaccine entrapment in liposomes." METHODS, (1999 SEP) 19 (1) 156-62. REF: 29 , XP000973867 page 157 -page 160; table 2	1-5, 7-17,19, 20
X	PERRIE, YVONNE ET AL: "Genetic immunization using liposome-incorporated DNA" J. PHARM. PHARMACOL. (1998), 50(SUPPL., BRITISH PHARMACEUTICAL CONFERENCE 1998), 103 , XP000974033 the whole document	1-17,19

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Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

8 document member of the same patent family

Date of the actual completion of the international search

13 February 2001

Date of mailing of the international search report

22.02.01

Name and mailing address of the ISA

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Authorized officer

Mennessier, T

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GREGORIADIS G ET AL: "LIPOSOME-MEDIATED DNA VACCINATION" FEBS LETTERS,NL,ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, vol. 402, no. 2/03, 1997, pages 107-110, XP000872926 ISSN: 0014-5793 the whole document	1-5, -7-17,19
X	WO 98 10748 A (GREGORIADIS GREGORY ;UNIV LONDON PHARMACY (GB)) 19 March 1998 (1998-03-19) cited in the application page 5, line 28-37 page 10, line 3-5 page 13, line 16-20 page 25; table 3 page 26; table 4 page 27; table 5 page 34; table 7 claims 12,29,30	1-20
A	HAN M ET AL: "APPLICATION OF LIPOSOMES FOR DEVELOPMENT OF ORAL VACCINES: STUDY OF IN VITRO STABILITY OF LIPOSOMES AND ANTIBODY RESPONSE TO ANTIGEN ASSOCIATED WITH LIPOSOMES AFTER ORAL IMMUNIZATION" JOURNAL OF VETERINARY MEDICAL SCIENCE - NIHON JUIGAKU ZASSHI,JP,JAPANESE SOCIETY OF VETERINARY SCIENCE, TOKYO, vol. 59, no. 12, December 1997 (1997-12), pages 1109-1114, XP000872657 ISSN: 0916-7250 the whole document	1-20

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9810748 A	19-03-1998	AU 4215497 A	02-04-1998
		CN 1237102 A	01-12-1999
		EP 0938298 A	01-09-1999

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
12 April 2001 (12.04.2001)

PCT

(10) International Publication Number
WO 01/24773 A1

- (51) International Patent Classification⁷: **A61K 9/127**, 48/00, C12N 15/88
- (21) International Application Number: **PCT/GB00/03773**
- (22) International Filing Date: **2 October 2000 (02.10.2000)**
- (25) Filing Language: **English**
- (26) Publication Language: **English**
- (30) Priority Data:
99307786.6 **1 October 1999 (01.10.1999)** **EP**
- (71) Applicant (*for all designated States except US*):
LIPOXEN LIMITED [GB/GB]; 1 Portland Place,
London W1N 3AA (GB).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): **GREGORIADIS**, Gregory [CA/GB]; Lipoxen Limited, 2d Wimpole Street, London W1N 7AA (GB). **PERRIE**, Yvonne [GB/GB]; School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET (GB).
- (74) Agent: **GILL JENNINGS & EVERY**; Broadgate House, 7 Eldon Street, London EC2M 7LH (GB).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:**
- *With international search report.*
 - *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: **LIPOSOME-ENTRAPPED DNA ORAL VACCINES**

(57) Abstract: An oral vaccine comprises liposomes and complexed or, preferably, entrapped DNA operatively encoding an antigen, in which the liposomes are formed from components including cationic compounds and zwitterionic phospholipids. The hydrophobic groups within the liposome forming compounds must include at least one group which is saturated. This is believed to raise the transition temperature, rendering the liposomes more stable when delivered orally. The compositions have been found to give detectable increased in IgA levels, secreted immunoglobulins of importance in efficacious oral vaccine delivery.

WO 01/24773 A1

LIPOSOME-ENTRAPPED DNA ORAL VACCINES

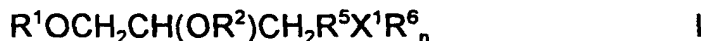
The present invention relates to oral vaccines comprising cationic liposomes and, complexed or entrapped within the liposomes, a gene vaccine, that is a nucleic acid coding for an antigen against which
 5 vaccination is desired.

In WO-A-9810748 gene vaccines are described comprising nucleic acid encoding antigen against which vaccination is required, in which the nucleic acid is entrapped within the liposomes. The liposomes are formed from liposome forming components including cationic lipid. The
 10 compositions are said to be suitable for administration by, *inter alia*, oral routes but in the examples, the compositions are administered intramuscularly, subcutaneously, intravenously or intraperitoneally.

For a vaccine to generate an immune response following oral administration, the composition must interact with the lymphoid system in the
 15 gut. The vaccine must consequently be stable in the GI tract, and must be stable enough to interact with the relevant cells of the system before being destroyed by bile salts. Clearly it is desirable for vaccines to be administratable orally rather than having to be injected. The present invention relates to compositions which are suitable for oral administration
 20 and to oral vaccines and methods for vaccinating human or non human animals by oral administration of the vaccines.

According to a first aspect of the invention there is provided a novel vaccine comprising a nucleic acid operatively encoding an antigen complexed with and/or entrapped within liposomes formed from liposome
 25 forming components including

a) at least one cationic compound having the general formula I,



in which R^1 and R^2 are the same or different and are selected from groups of the formula $CH_3(CH_2)_a(CH=CH-CH_2)_b(CH_2)_c(CO)_d-$

30 in which b is 0 to 6, a and c are each selected from 0-23 and $(a + c + 3b)$ is in the range 12-23 and d is 0 or 1;

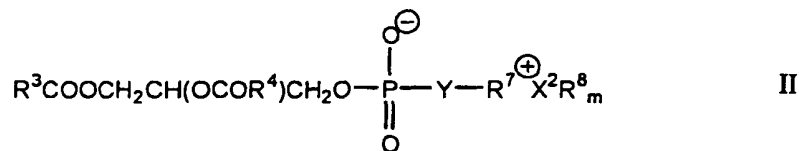
R^5 is a bond or a C_{1-8} alkanediyl group a C_{1-4} alkoxy - C_{1-4} alkyl group, or a C_{1-8} oxy-alkylene group ;

X^1 is N, P or S;

n is 3 where X^1 is N or P and is 2 where X^1 is S; and

5 the groups R^6 are the same or different and are selected from hydrogen, C_{1-8} alkyl, C_{6-12} aryl or aralkyl, or two or three of the groups R^6 together with X^1 may form a saturated or unsaturated heterocyclic group having 5 to 7 ring atoms;

b) at least one zwitterionic phospholipid having the general
10 formula II



15 in which R^3 and R^4 are the same or different and are selected from groups of the formula $CH_3(CH_2)_e(CH=CH-CH_2)_f(CH_2)_g-$

in which f is 0 to 6, each of e and g are 0 to 23 and (e + g + 3f) is in the range 12 to 23;

R^7 is a C_{1-8} alkanediyl group;

20 Y is -O- or a bond;

X^2 is N, P or S;

m is 3 when X^2 is N or P and is 2 when X^2 is S; and

the groups R^8 are the same or different and are selected from the group consisting of hydrogen, C_{1-8} alkyl, C_{6-11} aryl or aralkyl, or two or three
25 of the groups R^8 together with X^2 may form a saturated or unsaturated heterocyclic group having 5 to 7 ring atoms;

provided that in at least one of the groups R^1 , R^2 , R^3 and R^4 , b or f, as the case may be, is 0.

The composition is preferably an oral vaccine and the invention also
30 covers methods of administering the vaccine by oral routes. The composition may comprise pharmaceutically acceptable diluents, and may

include components to enhance the immunogenic properties of the vaccine, such as conventional adjuvants.

In the invention the proviso that at least one of the groups R^1 , R^2 , R^3 and R^4 should have an saturated long chain alkyl group tends to provide a composition which has a relatively high transition temperature. Thus the liposome forming components, in admixture, should have a transition temperature of at least 37°C, preferably in the range 38 to 50°C.

It is preferred that the groups R^1 and R^2 are the same as one another and that the groups R^3 and R^4 are the same as one another. In general the present inventors have found that it is desirable that either R^1 and R^2 are unsaturated and R^3 and R^4 are saturated, or vice versa. Preferably the cationic compound comprises a single compound of the formula I.

In a particular embodiment of the invention two zwitterionic phospholipids having a different formula, each within formula II, are used in the liposome forming components.

In one embodiment wherein such a mixture is used, in a first zwitterionic phospholipid, the groups R^3 and R^4 are the same and each represent a group, which f is 1, and in which $e + g$ is in the range 14 to 20, preferably in the range 14 to 18. Preferably the unsaturated group is midway along R^3 or R^4 that is $e \approx g$, preferably $e = g = 7$. Usually the ethylenic bond is *cis*.

In a second embodiment wherein a mixture of phospholipids is used in the first phospholipid of a mixture, the groups R^8 are preferably all the same and are preferably hydrogen. In the second phospholipid of the formula II, the groups R^8 are all the same and are C_{1-4} -alkyl. Often in this embodiment, for both phospholipids, f is 0.

Generally, in both embodiments using mixtures of phospholipids in both first and second phospholipids, Y is O and X^2 is N. Furthermore R^7 is preferably $C_{2,3}$ -alkanediyl.

In the cationic compound of the formula I, the hydrophobic groups R^1 and R^2 may be joined to the rest of the molecule through ether linkages (that is d is 0) or ester linkages (in which d is 1). Preferably in compounds of the

formula I, R⁵ is C₁₋₄-alkanediyl. Preferably the cationic compound is permanently cationic, that is substantially fully ionised at all pH's likely to be encountered *in vivo*, in the range 5 to 9. Preferably each of the group R⁶ is other than hydrogen, therefore, especially C₁₋₄-alkyl, most preferably each
5 group R⁶ being methyl.

R⁵ is preferably a bond or a methylene group.

A particularly preferred embodiment of the composition of the invention utilises a cationic compound of the general formula I in which each of the groups R¹ and R² is an oleoyl group, and in which the group R⁵ is a
10 bond, X¹ is N and each of the groups R⁶ is methyl (1, 2-bis(oleoyloxy)-3-(trimethylammonio)propane(DOTAP)). An alternative cationic compound is the analogous compound in the which the hydrophobic oleoyl groups are replaced by oleyl groups i.e. joined through ether linkages rather than ester linkages. A suitable cationic compound in which the hydrophobic groups are
15 saturated is 1, 2-bis(hexadecyloxy)-3-trimethylammino propane(BisHOP).

Suitable zwitterionic phospholipids include dioleoyloxy phosphatidyl ethanolamine (DOPE), dioleoyloxy phosphatidylcholine (DOPC), distearoyl phosphatidyl ethanolamine (DSPE), distearoyloxy phosphatidylcholine (DSPC), dipalmitoyl phosphatidyl ethanolamine (DPPE), dipalmitoyl
20 phosphatidylcholine (DPPC), and admixtures. A particularly preferred zwitterionic phospholipid mixture comprises distearoyl phosphatidylcholine and dioleoyl phosphatidyl ethanolamine.

A mixture of two zwitterionic phospholipids generally comprises the two compounds in weight ratios in the range 10:1 to 1:10, most preferably in
25 the range 5:1 to 1:5, more preferably 2:1 to 1:2. Preferably the proportion of groups R³ and R⁴ which are saturated in a mixture is at least 50%.

Generally the ratio of cationic compound to zwitterionic phospholipid (total) is in the range 10:1 to 1:20, more preferably in the range 5:1 to 1:10, more preferably in the range 1:1 to 1:5.

30 According to a further aspect of the invention there is provided an oral vaccine comprising a nucleic acid encoding an antigen complexed to and/or entrapped within liposomes formed from liposome forming components

including at least one glycerolipid, at least one cationic compound and at least one zwitterionic phospholipid characterised in that the glycerolipid is an O,O'-dialkanoyl or O,O'-dialkyl phospholipid. Preferably the glycerolipid is a compound of the general formula II above in which, in both R³ and R⁴ f is O.

5 In all aspects of the invention it is preferred that the liposome forming components in combination have a transition temperature of at least 37°C. Transition temperatures are determined by differential scanning calorimetry.

In this aspect of the invention the zwitterionic phospholipids preferably comprise a mixture of lipids, for instance a mixture of saturated and unsaturated lipids, and/or a mixture of phosphatidylcholines and phosphatidylethanolamines.

The cationic compound is preferably a 2,3-di(acyloxy or alkoxy) substituted propylamine derivative, for instance having the general formula I above. Alternatively the compound may be formed of simple cationic amphiphilic compounds such as mono- or di- stearylamine or other long chain alkyl amine, or the secondary, tertiary or quaternary derivatives thereof having, respectively, one, two or three N-lower alkyl (C₁₋₄ alkyl) substituents, such as dimethyldioctadecyl ammonium halides. Another category of amphiphilic cationic compounds which are suitable for incorporating into liposomes, is spermine conjugates with di(fatty acyl) glycerides or N,N-di(C₁₂₋₂₄) alkyl acyl amide compounds or 3β-[N-(N',N'-dimethylaminoethane)-carbonyl]cholesterol (DC chol). A range of suitable cationic amphiphilic compounds are described by Kabanov A.V. *et al* in Bioconjugate Chem. (1995), 6(1), 7-20, the content of which is incorporated herein by reference.

According to a further aspect of the invention there is provided an oral vaccine comprising a nucleic acid encoding an antigen complexed to and/or entrapped within liposomes formed from liposome forming components including at least one cationic compound and at least one zwitterionic phospholipid characterised in that the liposome forming components include at least 25 mole%, preferably at least 50 mole%, of components which individually have a transition temperature of more than 40°C.

In this aspect of the invention the effect of using relatively high levels of high transition temperature lipidic components is that the transition temperature of the mixture of liposome for using components will be above 37°C. The transition temperature of a mixture tends to be close to the averaged transition temperatures of the individual components. However it is generally easier to determine the transition temperature of individual components, the values for many of these being known. Preferred high transition temperature zwitterionic phospholipids are DPPC (T_c 41.4°C), DSPC (T_c 55.1°C), DPPE (T_c 64°C) and DSPE (T_c 74.2°C).

In all aspects of the invention other components may be included in the liposome forming mixture, such as cholesterol, in amounts up to 50% by weight. Preferably the liposome forming components are free of cholesterol.

The amount of cationic compound is preferably in the range 5 to 50% of the total moles of liposome forming components, preferably in the range 10 to 25% mole.

The liposome composition is generally in the form of an aqueous suspension for instance, a physiological buffer. Alternatively it could be a dried composition for rehydration.

The liposomes may be made by any of the generally used liposome forming techniques. The product liposomes may be multilamellar or unilamellar vesicles and may be relatively large (vesicle diameters in the range 300 nm to 2000 nm preferably with average diameters in the range 500-1000 nm), or small (vesicle diameters in the range 100 nm to 400 nm preferably with average diameters in the range 200 to 300 nm). Preferably the liposomes have a mean diameter not exceeding 1000 nm, and preferably substantially all have diameters less than 2000 nm. Most preferably the mean diameter is in the range 200-750 nm.

In the novel compositions the nucleic acid may be complexed with liposomes that is located externally of the liposomes. Preferably, however, the nucleic acid is at least partially entrapped.

Preferably the liposomes are formed by a process in which the vesicles are formed, mixed with nucleic acid to be entrapped and are then

dehydrated, preferably by freeze drying, and subsequently rehydrated in aqueous composition to make dehydration-rehydration vesicles (DRV's), optionally the DRV's may be subsequently subjected to microfluidization to reduce the average size. However, preferably the DRV's are not subjected to microfluidisation, or to only one or two cycles of microfluidisation. Preferably the non-entrapped material is separated from liposomes by centrifugation or molecular sieve chromatography, after the rehydration and/or microfluidization steps, although this may be unnecessary.

According to a further aspect of the present invention there is provided a method of entrapping polynucleotide into liposomes involving the steps of:

- i) forming an aqueous suspension comprising naked polynucleotide, which operatively encodes an immunogenic polypeptide useful to induce a desired immune response in a human or animal subject, and preformed liposomes formed of liposome forming components as specified for the novel compositions above,
- ii) freeze drying or spray drying the suspension, and
- iii) rehydrating the product of step ii) to form dehydration/rehydration vesicles.

Further steps which may be carried out but are not essential are:

- iv) subjecting the aqueous suspension of dehydration rehydration vesicles from step iii) to microfluidization to control the size; and/or
- v) optionally separating non entrapped polynucleotide from liposomes.

Step iv) is generally found to be unnecessary since the dehydration rehydration vesicles.

The last step is generally found to be unnecessary, since the external nucleic acid may be partially protected from the environment by being complexed to the cationically charged liposomes.

The dehydration-rehydration of steps are substantially as described by Kirby and Gregoriadis, (1984) *Biotechnology*, 2, 979-984, the content of which is incorporated herein by reference. Thus, the liposomes in step i) are preferably small unilamellar (SUV's) (although they may be MLV's for instance having size 2 μm) and made in step iii) are preferably multilamellar liposomes (MLV's) respectively. The product liposomes of step iii) are generally called dehydration-rehydration vesicles (DRV's).

Microfluidization of the DRV's is carried out substantially as described in WO-A-92/04009, the disclosure of which is incorporated herein by reference and by Gregoriadis et al, (1990), *Int. J. Pharm.* 65, 235-242. As mentioned above, if microfluidisation is conducted, it is preferred that no more than one of two cycles are conducted.

The present invention does not involve polymerising the liposome forming components to raise the transition temperature. This may reduce the delivery rate of active and is an undesirable extra step in the processing.

By using the DRV technique, inventors have established that up to 90% or even more of the polynucleotide present in the aqueous suspension subjected to the drying step can be entrapped into and/or complexed with the liposomes. The level of polynucleotide entrapment and/or complexing in the liposomal composition is preferably in the range 0.05 to 100, preferably 1 to 50, more preferably 5 to 50 $\mu\text{g}/\mu\text{mole lipid}$.

The liposome compositions of the invention have been found to be resistant to bile salts and this is believed to correlate with stability in the GI tract.

The nucleic acid active may be RNA, for instance which is directly transcribable and translatable in the synthesis of the antigen, or which must first be reverse transcribed to form DNA for replication. Preferably the nucleic acid is DNA which is preferably replicated, and is transcribed and translated to form the antigen of choice. The DNA is preferably a ds plasmid DNA.

The invention includes also the use of the compositions of liposomes or made by the processes of the invention in the manufacture of a

composition for use in a method of therapy or prophylaxis. For instance the method may be the immunisation (vaccination) of a human or animal subject to protect it against infection by infectious micro organisms. Alternatively an immune response may be generated by the gene product which is useful in immune therapy, for instance to treat cancer or other diseases, including infections.

The invention is illustrated further in the following examples:

Example 1

Methodology: Oral immunisation experiment 1

10 **Liposome preparation**

Liposomes with the following compositions were prepared using the Dehydration-Rehydration method (DRV);

- 1) 32 μ moles of egg phosphatidylcholine (PC), (mixture of di fatty acyl phosphatidylcholines, including some saturated groups)
15 16 μ moles of dioleoyl phosphatidylethanolamine (DOPE),
8 μ moles of dioleoyl trimethylammonium propane (DOTAP).
- 2) 32 μ moles of distearoyl phosphatidylcholine (DSPC),
16 μ moles of DOPE,
8 μ moles of DOTAP.
- 20 3) 32 μ moles of DSPC,
16 μ moles of cholesterol (CHOL),
8 μ moles of DOTAP.

600 μ g of pRc/CMV HBS plasmid DNA encoding for the S (small) region of Hepatitis B surface antigen (HBsAg; subtype ayw) was entrapped in the above liposome formulations using the following technique.

The dehydration-rehydration procedure (Kirby and Gregoriadis, (1984) *op. cit.*) was used for the incorporation of pRc/CMV HBS plasmid DNA into liposomes. In short, 2 ml of small unilamellar vesicles (SUV) were prepared from the specified liposome forming components mixed with plasmid DNA frozen at -20C and freeze-dried overnight. The liposomes were then subjected to controlled rehydration to generate multilamellar (Gregoriadis et al, (1993) *Biochim. Biophys. Acta* 1147, 185-193)

dehydration-rehydration vesicles (DRV). The product was not subjected to steps to remove non-entrapped DNA and probably includes external DNA complexed to the liposomes. No microfluidisation was conducted.

Entrapment complexation efficiency for each of the compositions was 85-95%, as determined by using ³⁵S-labelled DNA, produced from ³⁵S-dATP. The DRV's had mean diameters in the range 550 to 750nm.

Immunisation

The method is based on Roy, K. *et al* (1999) Nature Medicine 5(4) 387-391.

Groups of 4 female Balb/c mice (20-24g) were immunised orally with either "naked" (group 4) or liposome-entrapped (groups 1-3) DNA using animal feeding needles attached to a 1 ml syringe. Each mouse was fed with 100 µg of DNA in a volume of 500 µl of phosphate buffered saline (PBS) on days 0, 28 and 38.

Immunisation groups:

- 1) PC:DOPE:DOTAP (100 µg DNA) (invention)
- 2) DSPC:DOPE:DOTAP (100 µg DNA) (invention)
- 3) DSPC:CHOL:DOTAP (100 µg DNA) (invention)
- 4) "Naked" DNA (100 µg DNA) (reference)
- 5) Control (no DNA)

IgA extraction from foecal pellet

Foecal pellets were collected from the cages of mice on days 0, 14, 21, 32, 40, 48, 62, 84, 96 and 119.

These pellets were suspended in PBS at a concentration of 100 mg/ml, subjected to centrifugation and the supernatant (containing IgA) was analysed.

ELISA measurements

ELISA was done on foecal extracts to measure secretory IgA. Plates were coated with the S (small) region of Hepatitis B surface antigen (HBsAg; subtype ayw), blocked with 1 % BSA to avoid nonspecific binding and then pellet extracts added in duplicate (undiluted). Horseradish peroxidase-

conjugated goat anti-mouse IgA was added, followed by o-phenylenediamine substrate. Absorbance at 450nm was measured. Results in Figures 1a - i represent mean of duplicate measurements for each group of mice.

Example 2

5 Methodology: Oral immunisation experiment 2

Liposome preparation

Liposomes with the following compositions were prepared using the Dehydration-Rehydration method (DRV):

- 10 1) 32 μ moles of DSPC,
 16 μ moles of DOPE,
 8 μ moles of DOTAP.
- 2) 32 μ moles of DSPC,
 16 μ moles of distearoyl phosphatidylethanolamine (DSPE),
 8 μ moles of DOTAP.
- 15 3) 32 μ moles of DSPC,
 16 μ moles of dipalmitoyl phosphatidylcholine (DPPE),
 8 μ moles of DOTAP.
- 4) 32 μ moles of DSPC,
 16 μ moles of DOPE.

20 pRc/CMV HBS plasmid DNA was entrapped into the above liposome formulations using the same method as Example 1. DRV compositions 1, 2 and 3 entrapped 85 - 95% of the total amount of DNA used. The non-cationic DRV liposomes (composition 4) had an entrapment efficiency of 45-55% (of the total amount of DNA used). The DRV liposome sizes were in
25 the same range as in Example 1.

Immunisation

Groups of 4 female Balb/c mice (20-24g) were immunised orally with either "naked" (group 6) or liposome-entrapped (groups 1-5) DNA using animal feeding needles attached to a 1 ml syringe. Each mouse was fed
30 with either 50 μ g (group 5) or 100 μ g (groups 1,2,3,4 and 6) of DNA in a volume of 500 μ l of PBS on days 0, 32.

Immunisation groups:

- 1) DSPC:DOPE:DOTAP (100 µg DNA) (invention)
- 2) DSPC:DSPE:DOTAP (100 µg DNA) (invention)
- 3) DSPC:DPPE:DOTAP (100 µg DNA) (invention)
- 5 4) DSPC:DOPE (100 µg DNA) (reference)
- 5) DSPC:DOPE:DOTAP (50 µg DNA) (invention)
- 6) "Naked" DNA (100 µg DNA)
- 7) Control (no DNA)

IgA extraction from foecal pellet

10 Foecal pellets were collected from the cages of mice on days 0, 42, 55, 65 and 92. These pellets were suspended in PBS at a concentration of 100 mg/ml, subjected to centrifugation and the supernatant (containing IgA) was analysed.

ELISA measurements

15 ELISA was performed on fecal extracts to measure secretory IgA as for the first oral immunisation experiment. As for the first experiment, results in Figures 2 a-d represent the mean of duplicate measurements for each group of mice.

Oral immunisation experiment 3

20 This experiment aims to investigate further the influence of the liposome composition on liposome-mediated oral immunisation. Two factors were measured:

- 1) The influence of the combination of the presence of phosphatidylcholine and cholesterol in the bilayer.
- 25 2) The effect of substituting the cationic dioleoyl trimethylammonium propane with cholesterol 3β-N-(dimethyl-aminoethyl)carbamate (DC-Chol)

Methodology:**Liposome preparation**

30 Liposomes with the following compositions were prepared using the Dehydration-Rehydration method (DRV), as described above in Example 1.

- 1) 32 μ moles of phosphatidylcholine (PC),
16 μ moles of dioleoyl phosphatidylethanolamine (DOPE),
8 μ moles of dioleoyl trimethylammonium propane (DOTAP).
- 5 2) 32 μ moles of distearoyl phosphatidylcholine (DSPC),
16 μ moles of DOPE,
8 μ moles of DOTAP.
- 10 3) 32 μ moles of PC,
16 μ moles of cholesterol (CHOL),
8 μ moles of DOTAP.
- 15 4) 32 μ moles of DSPC
16 μ moles of cholesterol (CHOL)
8 μ moles of Cholesterol 3 β -N-(dimethyl-aminoethyl)carbamate
(DC-CHOL).

600 μ g of pRc/CMV HBS plasmid DNA encoding for the S (small) region of Hepatitis B surface antigen (HBsAg; subtype ayw) was entrapped in the above liposome formulations. Entrapment efficiency for each of the compositions was 85-95%. The DRV diameters were in the same range as in Example 1.

Immunisation

Groups of 4 female Blab/c mice (20-24g) were immunised orally with either "naked" (group 4) or liposome-entrapped (groups 1-4) DNA using animal feeding needles attached to a 1 ml syringe. Each mouse was fed with 100 μ g of DNA in a volume of 500 μ l of PBS on days 0, 28 and 38.

Immunisation groups:

- 1) PC:DOPE:DOTAP (100 μ g DNA)
- 2) DSPC:DOPE:DOTAP (100 μ g DNA)
- 30 3) PC:CHOL:DOTAP (100 μ g DNA)
- 4) DSPC:DOPE:DC-Chol (100 μ g DNA)

5) "Naked" DNA (100 µg DNA)

IgA extraction from fecal pellet

Fecal pellets were collected from the cages of mice on days 0, 30, 45, 60, 70.

5 These pellets were in PBS at a concentration of 100 mg/ml, subjected to centrifugation and the supernatant (containing IgA) was analysed.

ELISA measurements

ELISA was done on fecal extracts to measure secretory IgA. Plates were coated with the S (small) region of Hepatitis B surface antigen (HBsAg; subtype ayw), blocked with 1% BSA to avoid nonspecific binding and then pellet extracts added in duplicate (undiluted). Horseradish peroxidase-conjugated goat anti-mouse IgA was added, followed by o-phenylenediamine substrate. Absorbance at 450 nm was measured. Results represent mean of duplicate measurements for each group of mice.

15 **Results**

Excreted IgA immune responses measured 60 and 70 days after the first dose are shown in figs 1 and 2 respectively. Results show DRV composed DPSC:DOPE:DOTAP enhanced the highest responses in orally immunised mice at both time points. Replacement of the cationic lipid DOTAP with DC-CHOL in the liposome entrapped DNA results in lower anti-HBsAg IgA immune responses. Further, liposomes composed of PC:CHOL:DOTAP were also less effective than those composed of DSPC:DOPE:DOTAP in mediating immune responses.

Conclusions

25 The conclusions to be drawn from Examples 1 to 3 are that the experiments are repeatable. Furthermore it appears that relatively low levels of entrapped DNA provide adequate transfection rates for an immune response (comparing groups 1 and 5 of Example 2). The saturated lipids seem to produce liposomes having better performance.

Example 4

Reporter gene expression after oral dosing

Aim

To compare levels of gene expression in mesenteric lymph node after
5 oral dosing of mice with either naked or liposome-entrapped plasmid DNA
encoding fluorescent green protein reporter gene (pCMV.efgp). If the
reporter gene is expressed, as indicated by visible green protein in
recovered lymph nodes, this is an indication that the DNA reaches the
mesenteric lymph nodes and is there endocytosed and expressed. Antigen
10 presenting cells are located in the lymph nodes, the target for gene vaccines
to generate an immune response.

Methodology:

Liposome preparation

Liposomes composed of 32 μ moles of DSPC, 16 μ moles of DOPE, 8
15 μ moles of DOTAP were prepared using the Dehydration-Rehydration
method (DRV) as described for example 1 and 600 μ g of pCMV.efgp plasmid
DNA entrapped.

Dosing and measurement of gene expression

2 female Balb/c mice (20-24g) were dosed orally with either "naked"
20 or liposome-entrapped DNA using animal feeding needles attached to a 1 ml
syringe. Each mouse was fed with 100 μ g of DNA in a volume of 500 μ l of
PBS. 44 h after dosing, mesenteric lymph nodes were collected from dosed
and control (naive) mice. The freshly collected lymph nodes were adhered
to Cryostat chucks using Tissue-Teck (Miles Inc, USA), then frozen in liquid
25 nitrogen. Sections were cut at 20 μ m in a SLEE Cryostat. Images were
captured under Nikon microphoto Microscope, using incident fluorescence
and Kodak Ektachrome 4000 ASA.

Results and Conclusions

Higher levels of the plasmid encoded fluorescent green protein can
30 be seen in the mesenteric lymph nodes of mice dosed with liposome-
entrapped pCMV.efgp (Fig 4a) compared to those which received naked

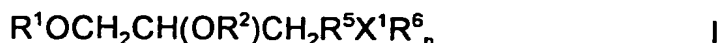
pCMV.efgp (Fig 3b) and background levels as shown in lymph node sections taken from naive mice (Fig 3c). From this it may be concluded that orally administered DNA is cleared to the mesenteric lymph node and that the rate of expression of reporter gene in the mesenteric lymph node is increased by

5 encapsulation in cationic liposomes comprising saturated lipids. This is consistent with the results showing the increase in immune response by entrapment in cationic liposomes formed from saturated lipids.

CLAIMS

1. An oral vaccine comprising a nucleic acid operatively encoding an antigen complexed with or entrapped within liposomes formed from liposome forming components including

5 a) at least one cationic compound having the general formula I,



in which R^1 and R^2 are the same or different and are selected from groups of the formula $CH_3(CH_2)_a(CH=CH-CH_2)_b(CH_2)_c(CO)_d-$

10 in which b is 0 to 6, a and c are each selected from 0-23 and (a + c + 3b) is in the range 12-23 and d is 0 or 1;

R^5 is a bond or a C_{1-8} alkanediyl group;

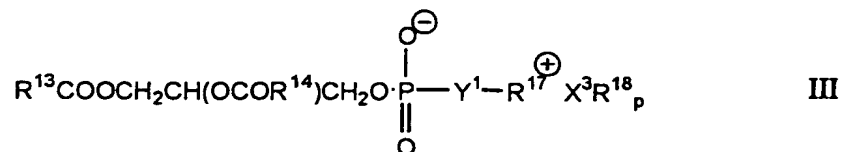
X^1 is N, P or S;

n is 3 where X^1 is N or P and is 2 where X^1 is S; and

15 the groups R^6 are the same or different and are selected from hydrogen, C_{1-8} alkyl, C_{6-12} aryl or aralkyl, or two or three of the groups R^6 together with X^1 may form a saturated or unsaturated heterocyclic group having 5 to 7 ring atoms;

b) at least one zwitteronic phospholipid having the general formula II

20



25 in which R^3 and R^4 are the same or different and are selected from groups of the formula $CH_3(CH_2)_e(CH=CH-CH_2)_f(CH_2)_g-$

in which f is 0 to 6, each of e and g are 0 to 23 and e + g + 3f is in the range 12 to 23;

R^7 is a C_{1-8} alkanediyl group;

Y is -O- or a bond;

30 X^2 is N, P or S;

m is 3 when X^2 is N or P and is 2 when X^2 is S; and

the groups R^8 are the same or different and are selected from the group consisting of hydrogen, C_{1-8} alkyl, C_{6-11} aryl or aralkyl, or two or three of the groups R^8 together with X^3 may form a saturated or unsaturated heterocyclic group having 5 to 7 ring atoms;

5 provided that in at least one of the groups R^1 , R^2 , R^3 and R^4 , b or f, as the case may be, is 0.

2. A vaccine according to claim 1 in which $R^1=R^2$ and $R^3=R^4$.

3. A vaccine according to claim 2 in which R^1 and R^2 represent a different group to R^3 and R^4 .

10 4. A vaccine according to claim 2 and claim 3 in which in R^1 and R^2 $b=1$ and in which $(a + c)$ is in the range 10-20.

5. A vaccine according to any of claims 2 to 4 in which $d = 0$.

6. A vaccine according to any of claims 2 to 5 in which $f = 0$.

7. A vaccine according to any preceding claim in which X^1 is N
15 and in which the R^6 groups are all C_{1-4} alkyl.

8. A vaccine according to any preceding claim which comprises two zwitterionic phospholipids each having the formula II, in which Y is O, and X^2 is N, and the groups R^8 of the first phospholipid are all hydrogen and the groups R^8 of the second phospholipid and all C_{1-4} alkyl, preferably
20 methyl.

9. A vaccine according to claim 8 in which, in each phospholipid Y is O and R^7 is $(CH_2)_h$ in which h is 2 or 3.

10. A vaccine according to claim 8 or claim 9 in which the groups R^3 and R^4 of the first phospholipid are the same and each is a group in which
25 $f=1$ and $(e + g)$ is in the range 10 to 20, preferably 12 to 14.

11. A vaccine according to any of claims 8 to 10 in which the groups R^3 and R^4 of the second phospholipid are the same and each is a group in which $f=0$ and $e + g$ is in the range 15 to 23, preferably 15-17.

12. An oral vaccine comprising a nucleic acid encoding an antigen
30 complexed to or entrapped within liposomes formed from liposome forming components including at least one glycerolipid, at least one cationic

compound and at least one zwitterionic phospholipid characterised in that at least one glycerolipid is an O'O-dialkanoyl or O,O'-dialkyl phospholipid.

13. A vaccine according to claim 12 in which the glycerolipid is a compound of the general formula II defined in claim 1 in which f is 0 in both
5 R³ and R⁴.

14. An oral vaccine comprising a nucleic acid encoding an antigen complexed to and/or entrapped within liposomes formed from liposome forming components including at least one cationic compound and at least one zwitterionic phospholipid characterised in that the liposome forming
10 components include at least 25 mole%, preferably at least 50 mole%, of components which individually have a transition temperature of more than 40°C.

15. A vaccine according to any of claims 12 to 14 in which the zwitterionic phospholipid is selected from the group consisting of
15 distearoylphosphatidylcholine, distearoylphosphatidylethanolamine, dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylethanolamine and mixtures thereof.

16. A vaccine according to any of claims 12 to 15 in which the cationic compound is a compound of the general formula I as defined in
20 claim 1.

17. A vaccine according to any of claims 12 to 15 in which the cationic compound is DC-cholesterol.

18. A method in which a human or a non-human animal is vaccinated by administering a vaccine according to any preceding claim
25 orally whereby an immune response to the encoded antigen is generated.

19. A method of entrapping polynucleotide into liposomes involving the steps of:

i) forming an aqueous suspension comprising naked
polynucleotide, which operatively encodes an immunogenic
30 polypeptide useful to induce a desired immune response in a human or animal subject, and preformed liposomes formed of

liposome forming components as defined in claim 1, claim 12 or claim 14,

ii) freeze-drying or spray-drying the suspension, and

iii) rehydrating the product of step ii) to form

5

dehydration/rehydration vesicles.

20. A method according to claim 19 comprising the further steps of:

iv) subjecting the aqueous suspension of dehydration/rehydration vesicles from step iii to microfluidization to control their size;

and

10

v) optionally separating non entrapped polynucleotide from liposomes.

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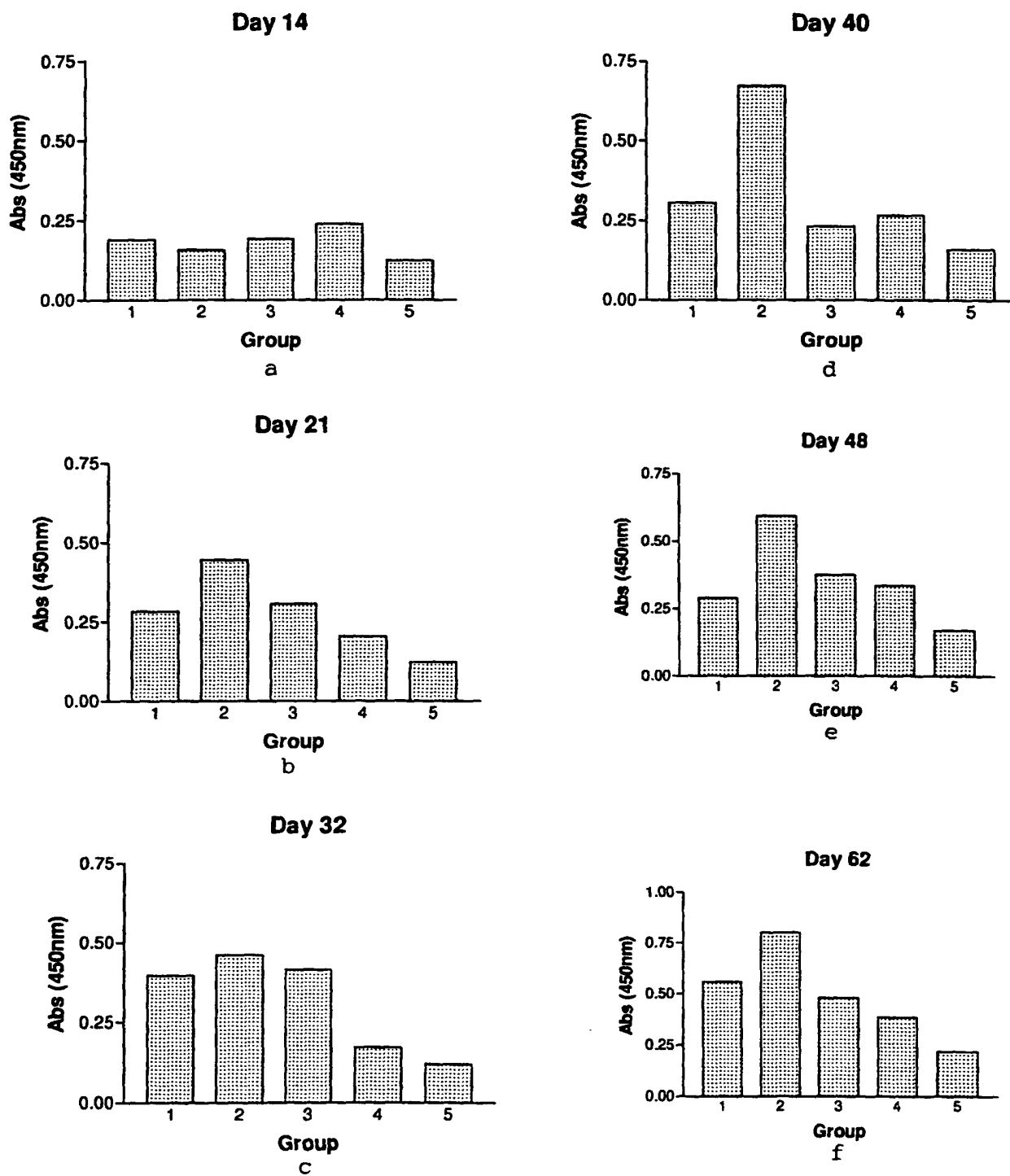


Fig. 1

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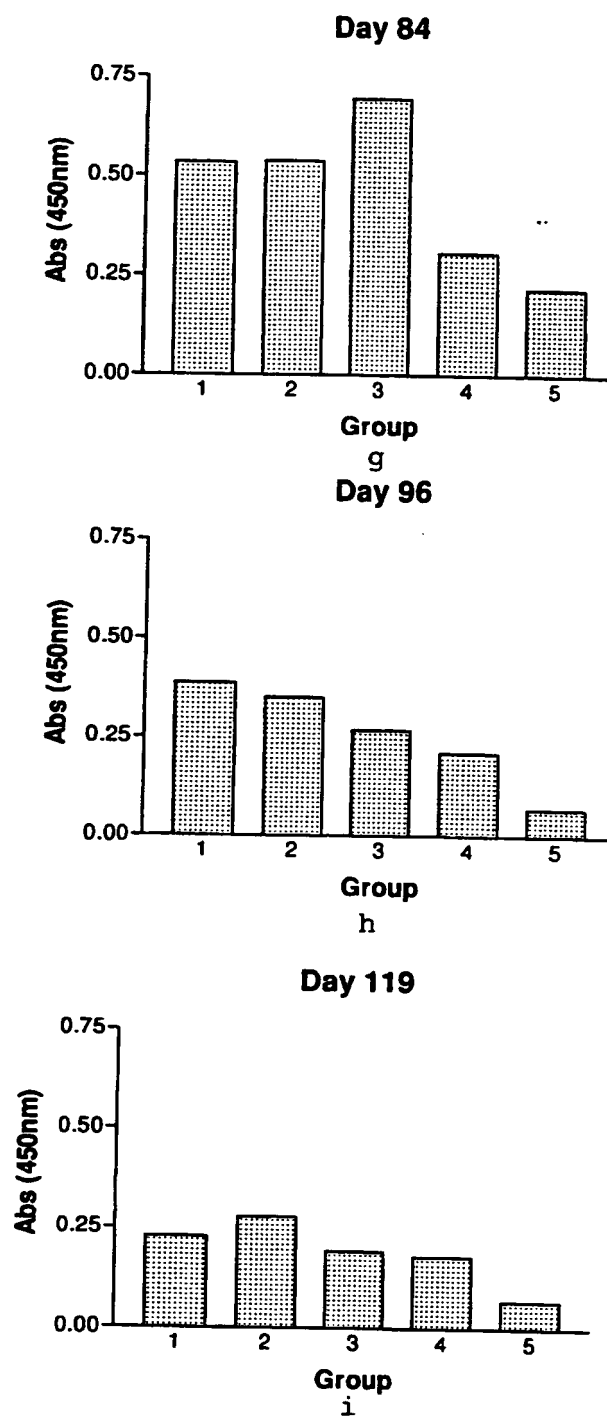


Fig. 1 cont.....

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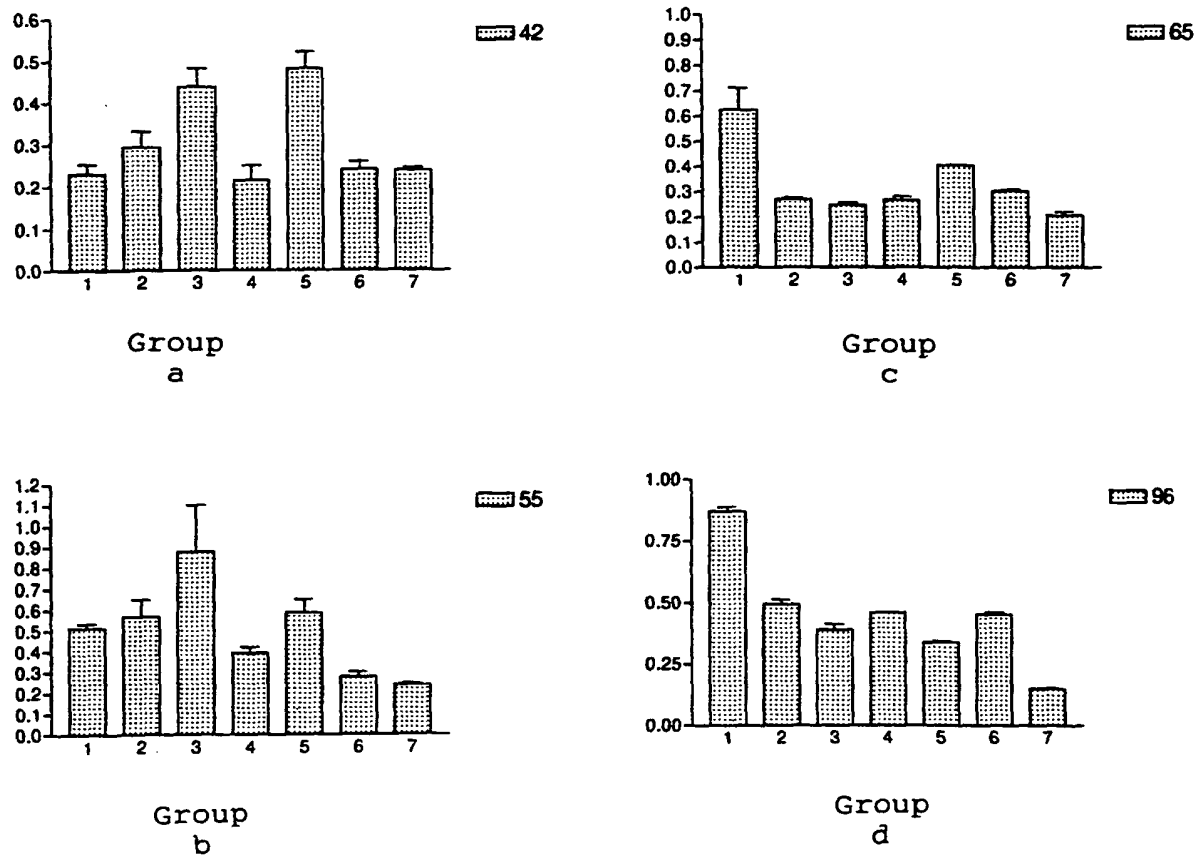
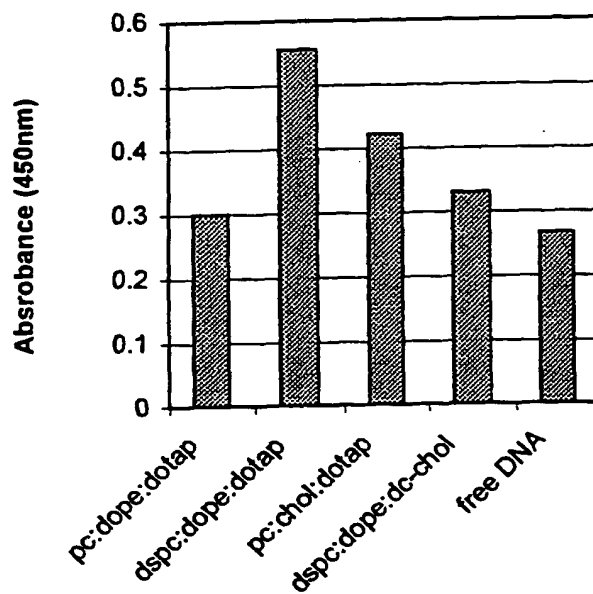


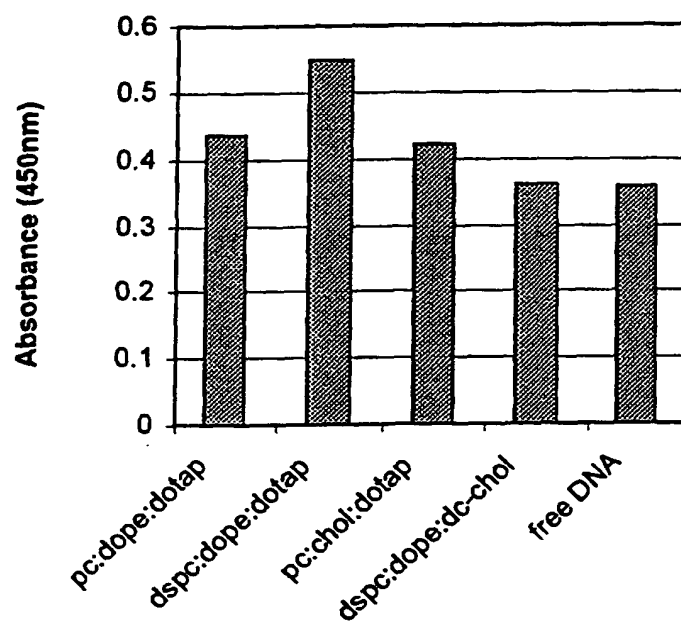
Fig. 2

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60 days

Fig. 3a



70 days

Fig. 3b

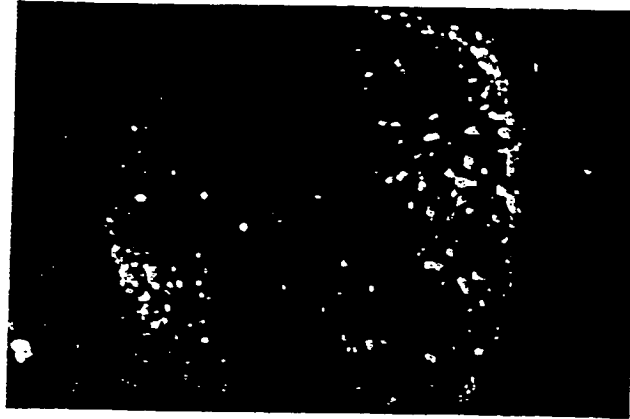


Fig. 4a



Fig. 4b



Fig. 4c